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BENZENE, 1,3-DIISOCYANATOMETHYL (26471-62-5)		

A 02

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Attn: 8(d) HEALTH & SAFETY STUDY REPORTING RULE
(REPORTING)

Dear Sir or Madam:

We herewith submit a copy of the following recently completed health and safety study.

"EVALUATION OF IN VITRO PRODUCTION OF THE B CHEMOKINE<MCP -1, AS A DIAGNOSTIC TEST IN DIISOCYANATE ASTHMA."

Name of Chemical Substance:	benzene 1,3-diisocyanatomethyl-
Common name:	generic toluene diisocyanate
Chemical Abstracts Service Number:	26471-62-5
Abbreviation:	2,4-TDI and 2,6-TDI (mixture)

Name of Chemical Substance:	benzene, 1,1'—methylenebis[isocyanato-
Common name:	generic MDI
Chemical Abstracts Service Number:	26447-40-5
Abbreviation:	MDI

Authors: David I. Bernstein, Zana Lummus, I. Leonard Bernstein, Andre Cartier, Jean-Luc Malo, and Louis-Phillippe Boulet

19216

The International Isocyanate Institute (III) project identification number (11331) has been marked on the title page of the report. Please refer to the III identification number in any communication regarding this study. The enclosed report does not contain any Confidential Business Information.

This study was sponsored by the International Isocyanate Institute on behalf of the following:

The Dow Chemical Company
Bayer Corporation
BASF Corporation
ICI Americas, Inc.
Lyondell Chemical Company

Very truly yours,

M.J. Blankenship
Managing Director

Enclosure: Study

A 05
III Project 147

11331

FINAL REPORT

International Isocyanate Institute Project

EVALUATION OF IN VITRO PRODUCTION OF THE β CHEMOKINE, MCP-1, AS A DIAGNOSTIC TEST IN DIISOCYANATE ASTHMA

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Introduction

The primary aim of this proposal is to validate diisocyanate (DIISO) specific in vitro cellular MCP-1 as a diagnostic marker of occupational asthma (OA) by using the specific bronchoprovocation test (SBPT) as the gold standard for diagnosis. A secondary aim of the study is to evaluate the sensitivity and specificity of in vitro serum DIISO-HSA specific IgG and IgE responses for identification of diisocyanate asthma (DA) confirmed by the SBPT. This report summarizes and discusses the results obtained in this study.

Clinical Methods

Subjects. A pilot study was conducted in 3 groups including: *Group I)* formerly exposed DIISO workers in whom a diagnosis of diisocyanate asthma was confirmed by a SBPT with the diisocyanate chemical encountered at work ; *Group II)* a group of formerly exposed workers without DA, excluded by a negative SBPT; *and Group III*) a control group of volunteers with no exposure to diisocyanates. All subjects were successfully recruited from the occupational respiratory clinics of Drs. Malo and Cartier at the Hopital Sacre-Couer in Montreal, Canada and of Dr. Boulet at Hopital Laval in Quebec.

Medical evaluation. All subjects underwent complete medical and occupational histories. The exact nature and duration of exposure to various agents at work was obtained. Methacholine testing was performed in each subject as was baseline spirometry testing. Single blinded placebo controlled inhalation challenge tests with workplace relevant isocyanate agents were administered. A positive response was defined as a greater than 20% decrease in FEV_1 from pre-challenge baseline. Venipuncture was performed to obtain 60-100 cc of peripheral blood which was anticoagulated via acid citrate buffer. Samples were immediately packaged and shipped overnight from Canada to the University of Cincinnati Allergy Laboratory for immediate processing by separation of peripheral blood mononuclear cells (PBMCs) and placement in tissue culture media with diisocyanate-HSA conjugate antigens and control antigens. Preliminary studies indicated that 24 hour shipping had no significant effect on antigen stimulated in vitro MCP-1 production. Methods are described below:

Laboratory Methods

Preparation of diisocyanate-human serum albumin antigens. Hexamethylene diisocyanate (HDI)-, methylene diphenyl diisocyanate (MDI)-, and toluene diisocyanate (TDI)- conjugated human serum albumin (HSA) antigens (HDI-HSA, MDI-HSA, TDI-HSA) were prepared and characterized as previously described (1, 2). Typically, diisocyanate antigens contain 2-13 moles of isocyanate per mole of protein.

In vitro stimulation of PBMCs. Mononuclear cells purified from whole blood consist of 81 - 89 % lymphocytes, 9 - 14 % monocytes, 2 - 5 % granulocytes, 0 - <7 % eosinophils, < 2 % basophils, and negligible platelets and red cells. Cells are suspended in RPMI medium

containing 5 % heat inactivated FBS as previously described and plated at a cell concentration of 5 x 10^6/ml. and incubated with HBSS, PHA, HSA, TDI-HSA, MDI-HSA, or HDI-HSA. The mitogen, PHA, a non-specific activator of PBMCs served as the positive control reagent. After 48 hours incubation, 37°, 5% CO_2, supernatants are removed and stored at -80 °C until assayed (3,4).

Immunochemical assay for MCP-1. A commercial immunoassay were used to quantitate MCP-1. Data was analyzed for antigen induced MCP-1 synthesis. Spontaneous production (media alone) and HSA enhanced production of MCP-1 by PBMCs was also assessed. A positive MCP-1 response is defined as supernatant levels that are 3 standard deviations above the mean MCP-1 obtained from antigen stimulated cultures of a reference group (n=8) of non-diisocyanate exposed subjects without asthma.

Specific anti-diisocyanate-HSA serum antibodies. Specific IgE and IgG are assayed in all subjects. High protein binding ELISA plates are coated with 10 µg/ml of protein antigen. Ten non-DIISO exposed control sera, a positive reference serum and patient sera are added to the plate at dilutions of 1:10 and 1:100. IgE antibodies are measured by a sandwich indirect ELISA, using unlabeled goat anti-human IgE, followed by alkaline phosphatase labeled rabbit anti-goat immunoglobulins (5). IgG antibodies are measured by standard indirect ELISA. A kinetic assay procedure is used, in which all reactions were terminated with 1 N NaOH when a standard positive control serum shows an $OD_{405\ nm}$ of 0.6. Sera are considered positive at an OD reading of 3 standard deviations greater than the mean OD of 8 negative controls.

Data Analysis. The specific antibody and MCP-1 data were analyzed as categorical data. Characteristics of sensitivity and specificity were determined for the in vitro MCP-1 assay. MCP-1 results between the 3 groups were also compared by one way ANOVA.

RESULTS

Antibody Studies

Immunoassays were performed in 18 diisocyanate workers exposed to one of three diisocyanate chemicals [TDI (n=4); MDI (n=5); HDI (n=9)]. Eight subjects were SBPT positive and 10 were negative. Based on results of the SBPT, sensitivity and/or specificity of elevated spIgE (3 SD ≥ control-mean and > OD of 0.1) were 63% and 100%, respectively. SpIgE antibody status was significantly associated with a positive SBPT (p=0.03). The predictive values of a positive and a negative spIgE test were 100% and 73%, respectively. The sensitivity and specificity of DIISO-HSA specific IgG were 63% and 78%, respectively. The mean duration of exposure prior to testing was longer in the SBPT positive (15±9.3) vs. SBPT negative (6.1±5 months) workers (p=0.02) but no difference was found between antibody positive and negative workers. This demonstrated that DIISO-antigen spIgE is highly specific in confirming DIISO asthma but lacks the sensitivity or negative predictive value required to exclude OA among DIISO workers. In contrast, spIgG had moderate sensitivity and a high frequency of false positive results. SpIgE was assayed 6-20 months after cessation of DIISO exposure validating the use of

this test in formerly exposed workers.

In vitro antigen stimulated MCP-1 production

These results are shown in table 1 and also in figure 1 and expressed as ng/ml of MCP-1 production at 48 hours after co-culture with antigens. Of 18 workers who underwent SBPT, 8 were positive to SBPT (Group 1) and 10 were SBPT negative (Group 2). Eight non-exposed controls (Group 3) were assayed for in vitro MCP-1 activity. The mitogen, PHA, was the positive control stimulator used to test the non-specific MCP-1 response of shipped cells. As shown in table 1, the the mean PHA response was 491 ng/ml in the SBPT+ group versus 322 ng/ml in the SBPT- group but the difference was not significant (p=0.10).

There was no significant difference between Groups 1,2 and 3 in the MCP-1 responses to individual diisocyanate-HSA antigens or to "work-relevant antigens" (i.e., the MCP-1 response produced by in vitro stimulation with the antigen prepared from the identical chemical to which each subject was exposed to at work). Therefore, MCP-1 results were analyzed as the "maximal response" to any of a panel of 3 antigens prepared in our laboratory (MDI-HSA, TDI-HSA and HDI-HSA) (see table 1). In table 1, data is shown in only a small number of subjects for MCP-1 responses obtained with diisocyanate-HSA conjugates prepared in the laboratory of Drs. William Brown and Amy Kennedy (TDI55, TDI56, TDI61, MDI67 and MDI68). Due to the limited numbers of purified PBMCs available for each experiment, it was not possible to conduct experiments in every subject with the latter antigens. Therefore, there was not adequate data to compare the performance of antigens prepared in different laboratories.

Because background MCP-1 responses to HSA and media were minimal, and did not affect overall results, the data is expressed as the maximal diisocyanate-HSA induced MCP-1 without corrections for media or HSA alone. Kruskal-Wallis one way ANOVA demonstrated a significant difference ($p < 0.05$) between Group 1 and Group 2 and Group 1 and Group 3. Figure 1 suggests that the MCP-1 assay was specific for identifying challenge positive workers at a level of MCP-1 above 300 ng/ml. There is overlap between groups 1 and 2 at levels $\leq$ 300 ng/ml. When a positive MCP-1 response is defined as $\geq$ 3 SD above the mean of Group 3 (161 ng/ml), all 8 workers who are SBPT positive (Group 1) were also MCP-1 positive as opposed to 3/10 challenge MCP-1 positive SBPT negative workers (Group 2). Thus, this assay exhibited 100% sensitivity and 71% specificity for identification of DA.

A sub-analysis was performed on those workers with active asthma in groups 1 and 2 confirmed by a positive methacholine test; this included 8 workers in Group 1 and 4 workers in Group 2. Figure 2 shows that there was clear separation of MCP-1 responses between diisocyanate asthma (Group 1) and asthmatic SBPT negative workers in group 2, which suggests that the assay could be more specific if applied primarily in the evaluation of asthmatic workers with a high pre-test probability of having DA. The test could be useful in discriminating asthma from non-OA in workers chronically exposed to diisocyanates.

SUMMARY AND CONCLUSIONS

In summary, these studies of workers with diisocyanate asthma (DA) demonstrated that in vitro production of MCP-1 by diisocyanate antigens was specifically increased in workers with diisocyanate asthma and not increased in isocyanate exposed workers with non-occupational asthma. In contrast to previous studies of MCP-1 in diisocyanate workers who were not confirmed via the SBPT, MCP-1 responses were not specific for the relevant isocyanate antigen (to which the workers had previously been exposed to) in the workplace. This could be explained by the possibility that epitopes providing optimal stimulation could be formed by a heterologous ligand-carrier protein conjugate. Thus, use of a panel of diisocyanate antigens in the MCP-1 test may yield optimal diagnostic sensitivity. The test may be more efficient if applied specifically to diisocyanate-exposed workers in whom a diagnosis of asthma has been objectively confirmed by a positive methacholine test or by clinical documentation of variable airflow obstruction (see figure 2).

Overall the results clearly indicate that in vitro diisocyanate enhancement of MCP-1 could be a useful diagnostic test for DA. However, the unequivocal validation of this cytokine assay for use in the routine evaluation of DA can only be achieved in an expanded study with larger groups of well characterized groups of diisocyanate-exposed asthmatic workers.

Antibody data confirmed results of other groups of investigators which showed that elevated serum specific IgE for MDI-HSA and HDI-HSA are specific tests which can be useful in confirming OA in a subset of affected workers but this assay lacks the necessary sensitivity needed to rule out a diagnosis of DA. Because different antibody assays are currently being used in the clinical evaluation of diisocyanate-exposed workers, further studies are needed to refine and standardize methods used to measure diisocyanate antigen specific IgG and IgE assays.

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Figure 1. MCP-1 response to any diisocyanate-HSA antigen

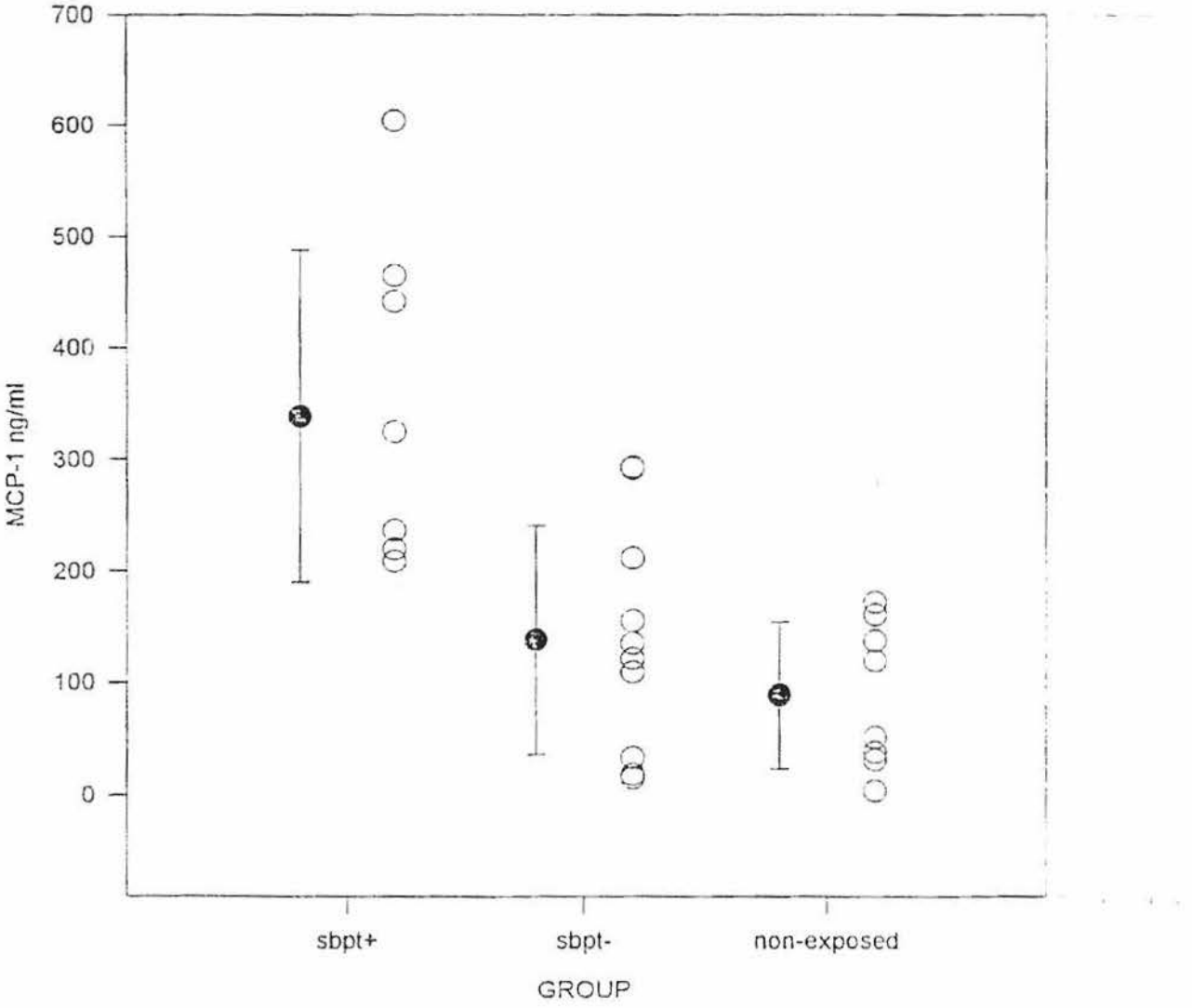

Figure 2. In vitro MCP-1 responses in workers with confirmed DA (n=8) and in diisocyanate exposed workers without DA (n=4).

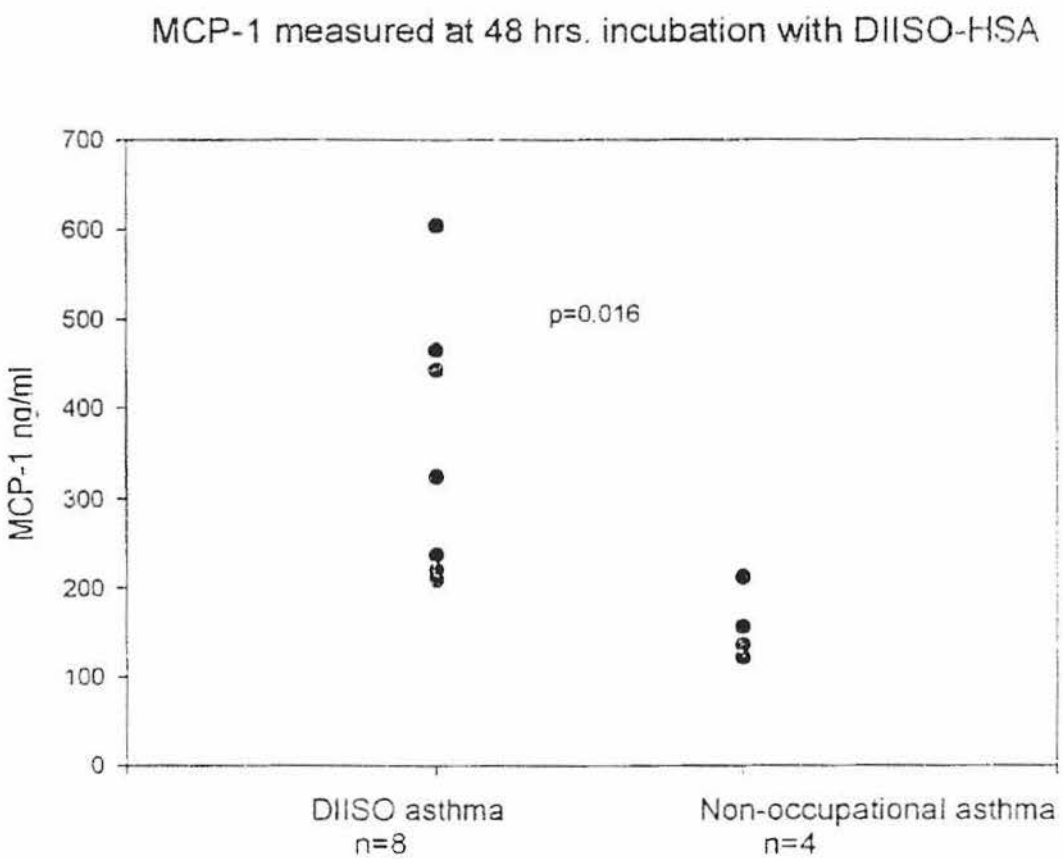

TABLE 1. MCP-1 ENHANCEMENT SUBJECTS 1-23

TABLE 1.																		
MCP1 in PBMC supernatants of III subjects 48 hour supes 50 ug stimulator/1ml ACPD cells																		
Comparison of maximum response observed to any diisocyanate																MAX ENHANCEMENT		
																		PHA/
subj #	SBPT +	n=6	medium	PHA	HSA-2	MDI-2	HDI-2	TDI-1	PA	TDI55	TDI56	TDI61	MDI67	MDI68	- med	-HSA	diiso
1	TDI	50	0	764	40	**442**	56	47	37						442	402	1.73
3	HDI	50	69	812	89	**237**	96	19	18						168	148	3.43
8	HDI	50	54	135	0	0	173	**209**	139						155	209	0.65
10	MDI	50	82	425	0	140	385	**465**	51		221			65	383	465	0.176
11	HDI	50	150	543	72	329	**604**	596	5		24			104	434	532	0.90
12	HDI	50	63	572	0	87	**325**	227	6						262	325	1.76
16	TDI	50	8	336	18	38	**209**	ND	22						201	191	1.61
17	MDI	50	15	344	0	17	**220**	ND	0						205	220	1.56
	MEAN		55.13	491.38	27.38	161.25	258.50	260.50	34.75		122.50			84.50	**283.75**	**311.50**	**1.43**
	S.D		49.06	227.90	35.92	160.65	176.66	228.94	45.51		139.30			27.58	123.55	141.77	0.98
	SBPT(-)	n=1	medium	PHA	HSA-2	MDI-2	HDI-2	TDI-1	PA	TDI55	TDI56	TDI61	MDI67	MDI68	- med	-HSA	diiso
2	HDI	50	10	348	72	0	10	**19**	10						9	-53	18.32
4	TDI	50	40	360	120	22	0	**122**	0	47	28	0	0	64	82	2	2.95
5	MDI	50	143	317	7	**292**	39	82	118	67	7	252	281	161	149	285	1.04
6	MDI	50	135	376	0	44	84	**110**	54	50	1	33	4	4	-25	110	3.42
7	HDI	50	73	297	60	64	116	**156**	74	133	106	95	56	68	83	96	1.90
13	HDI	50	169	272	125	80	**293**	96	129	313	160	97	124	334	125	168	0.93
14	HDI	50	33	424	4	16	100	**136**	17						67	96	4.24
20	MDI	50	41	571	33	**34**	nd	nd	52				22	37	-7	1	16.79
22	HDI	50	14	229	20	32	**212**	194	24	104	39	3	24	6	198	192	1.08
23			0.82	21.36	0.11	16.37	0.33	**16.41**	0.79	7.27	1.02	6.7	9.83	2.26	-0.49	0.22	64.73
	MEAN		**65.78**	**321.54**	**44.11**	**60.04**	**94.93**	**103.49**	**47.38**	**103.04**	**48.86**	**69.53**	**65.10**	**84.53**	**68.05**	**89.72**	**11.54**
	S.D		61.12	141.27	48.20	84.88	101.14	58.77	46.79	101.17	61.21	90.59	96.17	113.7	74.22	104.96	19.78
	NORMALS	n=6	medium	PHA	HSA-2	MDI-2	HDI-2	TDI-1	PA	TDI55	TDI56	TDI61	MDI67	MDI68	- med	-HSA	diiso
9	Boule	50	34	211	24	49	**172**	153	61						138	148	1.23
18	Cartie	50	5	212	0	0	**39**	nd	20						34	39	5.44
19	Cartie	50	0	331	0	0	**161**	nd	20						161	161	2.06
24	UC	50	0	390	0	0	**52**	25							52	52	7.50
25	UC	50	0	580	0	0	32	28	0						32	32	18.13
26	UC	50	0	500	0	0	0	**4**	0						4	4	125.0
27	UC	50	11	430	0	40	**120**	35							109	120	3.58
15	Boule	50	0	335	0	0	**138**	96	0						138	138	2.43
	MEAN		6.25	373.63	3	11.125	89.25	56.833	16.83						**83.5**	**86.75**	**20.67**
	S.D		24.04	87.68	16.97	34.65	24.04	40.31	43.13						0.00	7.07	0.85

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